EXPRESSION OF THE *BAR* AND *UIDA* GENES BY *GLADIOLUS* FOLLOWING THREE SEASONS OF DORMANCY

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Abstract

Gladiolus is propagated by corms that undergo dormancy each year, and at least three seasons of growth are required before the plants from tissue culture produce flowers. Successful genetic engineering of the flowering bulb crops will require long-term expression of transgenes. Transgenic *Gladiolus* plants containing the *bar-uidA* fusion gene under control by either the CaMV 35S, *rolD*, mannopine synthase (*mas2*), or ubiquitin (*UBQ3*) promoters were characterized for levels of GUS expression following three seasons of dormancy. The highest levels of GUS expression were found in callus, shoots, and roots of plants containing the CaMV 35S promoter as compared to shoots and roots from plants containing the *rolD*, *UBQ3*, and *mas2* promoters. Gene silencing of the *bar* and *uidA* genes did not occur in the 28 independently transformed plants analyzed following dormancy when both genes were expressed under the CaMV 35S, *rolD*, *mas2* and *UBQ3* promoters.

1. Introduction

Transgenic *Gladiolus* plants containing the *bar-uidA* fusion gene under 10 different promoters were developed and GUS expression was determined for plants regenerated and grown *in vitro* one year following bombardment (Kamo and Blowers, 1999; Kamo *et al.*, 2000). The plants that contained *bar-uidA* under either the CaMV 35S, *UBQ3*, *rolD* or *mas2* promoters were selected for a further long-term study because of their relatively high levels of GUS expression as compared to plants that contained the other six promoters (ubi3 and ubi7 from potato, translation elongation factor 1 subunit a, 2X CaMV 35S, actin, and PEP carboxylase) tested. This is the first report on long-term expression of a transgene in a floral bulb crop following three seasons of dormancy. Successful application of genetic engineering to floral bulb crops will require sustained transgene expression. Others have shown that loss and instability of transgenes can occur (Hanisch ten Cate *et al*., 1990; Fladung, 1999), but this did not occur in any of the transgenic *Gladiolus* plants, even after three years of dormancy.

2. Materials and methods

2.1. Plant material

Friable, embryogenic callus was initiated from *in vitro* grown plants of *Gladiolus* cv. Jenny Lee grown on Murashige and Skoog's (MS) basal salts medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and the following in mg l^{-1}: glycine, 1.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; 2,4-D, 0.5 and Gelrite, 0.2. Callus induction and multiplication required approximately 6 months. Suspension cell cultures initiated from the callus were grown in 20 ml of the same medium, with the omission of Gelrite, and subcultured once a week at a 1:1 dilution.

2.2. Development of transgenic plants

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Transgenic plants were developed as described (Kamo and Blowers, 1999; Kamo *et al*., 2000) and contained the *bar-uidA* fusion gene under control of either the CaMV 35S promoter (pDM327 received from D. McElroy), the *rolD* promoter (received from F. Leach, Center of Versaille Laboratorie de Biologie de la Rhizosphere, Cedex, France), the *UBQ3* promoter (received from J. Callis, University of California) or the *mas2* promoter (received from Mycogen Corp., San Diego, CA). The PDS-1000/He system (BioRad, Richmond, CA) was used for delivery of gold particles to suspension cells. One week after particle gun bombardment, cells were transferred to Gelrite solidified MS basal salts medium supplemented with 0.5 mg l^{1} 2,4-D and either 1 or 3 mg l^{1} bialaphos (Meiji Seika Kaisha, Tokyo, Japan). Cells were transferred to fresh medium that maintained the same concentration of bialaphos every 21 days and grown at 26°C in the dark. After approximately 6 months on selection medium, callus cells were transferred to regeneration medium consisting of MS basal salts supplemented with 2 mg l^{1} kinetin and the same concentration of bialaphos. Regenerating calluses were grown at 26°C under a 16 h light photoperiod.

2.3. GUS enzyme activity

Fluorometric determination of GUS enzyme activity was performed according to the method of Jefferson *et al*. (1987). Callus had been grown for 2 weeks on MS basal salts medium supplemented with 0.5 mg l^{1} 2,4-D without bialaphos when it was used for determination of GUS activity. The plant tissues were ground on ice in 500 µl extraction buffer (50 mM $NaH_2 PO_4$, pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, and 10 mM ß-mercaptoethanol) using a mortar and pestle followed by sonication for 5 min. Extracts were centrifuged at 10,000 x *g*, 4°C for 5 min, and an aliquot of the supernatant was added to the assay buffer (1 mM methylumbelliferyl- ß -D-glucuronide) for incubation. After incubation at 37°C an aliquot of the incubation mixture was added to 0.2 M sodium carbonate and the fluorescence measured using a BioRad VersaFluor fluorometer set at 360/40 nm for excitation and 460/10 nm for emission. Protein concentration was measured using the BCA (bicinchoninic) protein assay reagent (Pierce Co., Rockford, IL) according to the manufacturer's instructions.

Three calluses, each weighing approximately 100 mg fresh weight, were taken from three Petri plates for each transformed line. Callus had been induced from transgenic plants grown *in vitro* on MS basal salts medium supplemented with 0.5 mg l^{1} 2,4-D. Plants had been grown for 3 weeks on MS basal salts medium without bialaphos when tissue was collected for analysis. The basal 1 cm of each plant and 1 cm of the main root meristem were collected from three plants each grown in separate Magenta jars. Plants grown *in vitro* had undergone three seasons of dormancy that lasted 4 months. Nontransformed *Gladiolus* tissues of cv. Jenny Lee were used as the negative controls.

3. Results

3.1. Long-term comparison of promoter expression

All 28 transgenic plants analyzed continued to express the *uidA* gene that codes for GUS expression following three seasons of dormancy indicating that gene silencing did not occur when *uidA* was under either the CaMV 35S, *rolD*, *mas2*, or *UBQ3* promoters. Some of the plants expressed GUS in only the callus or roots whereas originally all plants expressed GUS in the shoots immediately following regeneration from callus. The highest levels of GUS expression were by callus, shoots, and roots that contained the CaMV 35S promoter, and the lowest levels were by plants containing either the *rolD* or *mas2* promoters.

The levels of expression in shoots from plants grown in vitro in 1996 cannot be directly compared to levels from plants grown in vitro in 1999 because of differences in sampling. The relative expression levels between the various plant lines analyzed in 1996 was in agreement with the relative expression levels for the plant lines in 1999. Plants lines that were relatively high expressors in 1996 continued their high level of expression until 1999.

3.2. Shoot and root expression

GUS expression under the *UBQ3* promoter was similar for shoots and roots. Shoots expressed at higher levels than roots when *uidA* was under the *rolD* promoter. Shoots containing the CaMV 35S promoter showed maximum levels of GUS expression that were higher than the highest levels expressed in shoots containing either the *rolD*, *UBQ3*, or *mas2* promoters. In contrast expression levels were higher for roots rather than shoots when *uidA* was under either the CaMV 35S or *mas2* promoters.

3.3. Callus expression

The levels of GUS expression in callus cells derived from the basal meristem of UBQ3 and CaMV 35S plants were approximately 3x that of shoots. Callus cells containing *rolD* expressed at levels less than that of shoots. Callus cells containing *UBQ3* expressed at approximately 10x higher levels than callus cells containing either the *rolD* or *mas2* promoters. The growth of callus on bialaphos did not affect levels of transgene expression.

4. Discussion

The level of GUS expression in transgenic plant tissues typically decreases with time, and silencing of *uidA* occurs frequently in progeny following meiosis. This preliminary study verified that loss or instability of transgenes is not a major problem in *Gladiolus*. Further studies are needed to determine how other environmental effects such as heat stress affect transgene expression by growing *Gladiolus* plants in the field. Inheritance of a transgene and its expression in the progeny following seed production remains to be confirmed, but at least three growing seasons are required before flowers are produced.

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